

## Development of a Triplex Real-Time PCR Assay for Detection of Panton-Valentine Leukocidin Toxin Genes in Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus*

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**Community-associated methicillin-resistant *Staphylococcus aureus* harboring Panton-Valentine leukocidin (PVL) genes is an emerging pathogen. A novel real-time PCR assay for identification of MRSA isolates containing PVL was developed. The PVL assay was used in a triplex format allowing simultaneous amplification of *mecA*, *nuc*, and PVL genes in 614 clinical isolates. This assay facilitates the rapid identification of PVL-positive isolates of MRSA.**

*Staphylococcus aureus* causes a range of infections, from mild skin infections to septicemia. Shortly after the introduction of methicillin in the early 1960s, methicillin-resistant *S. aureus* (MRSA) emerged and now causes serious nosocomial infections worldwide (1). Despite its spread in hospitals and nursing homes, MRSA has not disseminated in the wider community until recently (18). Such infections not acquired at a healthcare setting or institution are considered community-associated MRSA (CA-MRSA).

The discovery of Panton-Valentine leukocidin (PVL), encoded by *lukF-PV* and *lukS-PV* genes, was reported in the 1930s (16), and PVL has been associated with 1.6% of *S. aureus* strains in England and Wales in 2002 (8). Recently, MRSA strains producing PVL have been associated with an increase in severe skin infections and a new syndrome of community-associated necrotizing pneumonia in children (19). The PVL genes are carried on the phage  $\Phi$ SLT (15), but to date, PVL has not been identified with epidemic or endemic hospital-acquired MRSA strains (12).

With the emergence of the USA300 and USA400 CA-MRSA strains in Canada, also known as CMRSA10 and CMRSA7, respectively (13; M. Gilbert, J. Siushansian, J. MacDonald, D. Gregson, S. Elsayed, K. Zhang, K. Laupland, M. Louie, T. Louie, D. Nielsen, G. Keays, A. Honish, D. Gravel, M. Mulvey, J. Gillespie, and J. Conly, Abstr. AMMI Canada—CACMID 2005 Annu. Conf., abstr. G2, 2005), the challenge lies with laboratories to efficiently identify these strains for surveillance, infection control, and treatment protocols to limit the dissemination of CA-MRSA (11, 19, 20). Conventional PCR detection of the *lukF-PV* and *lukS-PV* genes of the PVL-encoding operon (17) allowed the identification of PVL-containing strains (11). However, real-time qualitative PCR de-

tection of PVL genes (6, 9, 14) can reduce the turnaround time significantly. In this report, we evaluated a high-throughput real-time PCR detection of PVL-encoding genes, multiplexed for simultaneous amplification of *mecA* (for detection of methicillin resistance) and *nuc* (for identification of *S. aureus*) (4). This triplex TaqMan assay is applied to the analysis of MRSA isolated from clinical specimens as part of a public health surveillance of MRSA.

Clinical MRSA isolates ( $n = 1,557$ ) were collected throughout Saskatchewan, Canada, primarily from skin and soft tissue infections from both healthcare and community settings between 1999 and 2005. All *S. aureus* isolates were considered oxacillin resistant by growth on Mueller-Hinton agar (containing oxacillin [6  $\mu$ g/ml] and 4% [wt/vol] NaCl) and by an automated sensitivity assay (Microscan; Dade Behring Canada, Inc., Mississauga, Ontario, Canada) (5) prior to PCR analysis. Nucleic acids from *S. aureus* were prepared by heating four to five colony picks from an overnight plate culture in 2% (wt/vol) Chelex 100 resin slurry (Bio-Rad Laboratories, Ltd., Mississauga, Ontario, Canada) for 10 min at 100°C. MRSA was confirmed by the detection of *mecA* and *nuc* by TaqMan duplex PCR or triplex PCR as described below. Primer and probe sequences for PVL (Table 1) were designed using Primer Express (ver. 2.0; Applied Biosystems, Mississauga, Ontario, Canada) and Oligo 6 (ver. 6.6.7.0; Molecular Biology Insights, Inc., Cascade, Colo.). A DNA alignment of publicly available *lukF-PV* and *lukS-PV* gene sequences from *S. aureus* and phage  $\Phi$ SLT (GenBank accession no. AB006796, X72700, AB009866, and AB045978) was considered to select a conserved target that spanned both genes of the operon. Specificity of primers and probe was assessed in silico against the NCBI (GenBank) nucleotide sequence databases by using BLASTN (2). Initially, PVL was detected with a single-target TaqMan PCR assay by using a dual-labeled probe with TET (6-carboxytetrachlorofluorescein) as the reporter and TAMRA (6-carboxytetramethylrhodamine) as the quencher (Applied Biosystems). Single-target PVL reactions were run simultaneously with the duplex assay for *mecA* and *nuc* in an ABI Prism 7700 sequence detector (Applied Biosystems) on the

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TABLE 1. PCR primer and probe sequences

Primer or probe name	Sequence (5'→3')	5' Reporter dye	Reaction concn (μM)
<i>mecA</i>			
<i>mecA</i> For	GGCAATATTACCGCACCTCA		0.30
<i>mecA</i> Rev	GTCTGCCACTTTTCCTTGT		0.30
<i>mecA</i> Probe	AGATCTTATGCAAACCTTAATTGGCAAATCC	FAM <sup>a</sup>	0.10
<i>nuc</i>			
<i>nuc</i> For	CAAAGCATCAAAAAGGTGTAGAGA		0.05
<i>nuc</i> Rev	TTCAATTTTCTTTCATTTTCTACCA		0.05
<i>nuc</i> Probe	TTTTTCGTAAATGCACTTGCTTCAGGACCA	VIC <sup>a</sup>	0.05
PVL			
PVL For	ACACACTATGGCAATAGTTATTT		0.30
PVL Rev	AAAGCAATGCAATTGATGTA		0.30
PVL Probe	ATTTGTAAACAGAAATTACACAGTTAAATATGA	TET <sup>a</sup> , Cy5 <sup>b,c</sup>	0.10
<i>luk-PV 1</i> <sup>d</sup>	ATCATTAGGTAAAATGTCTGGACATGATCCA		0.50
<i>luk-PV 2</i> <sup>d</sup>	GCATCAASTGTATTGGATAGCAAAAAGC		0.50

<sup>a</sup> Reporter dye quenched with 3' TAMRA quencher.

<sup>b</sup> Reporter dye quenched with nonfluorescent 3' BHQ3 quencher.

<sup>c</sup> PVL probe used for triplex PCR assay.

<sup>d</sup> PVL primers used for conventional PCR (11).

same plate, using a TaqMan PCR core reagent kit (Applied Biosystems) at 4.0 mM MgCl<sub>2</sub>. Thermal cycling was performed under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Data for the triplex PCR assay were collected with an ABI 7500 real-time PCR system (Applied Biosystems), with FAM (6-carboxyfluorescein) (*mecA*), VIC (*nuc*), and Cy5 (PVL) utilized as reporter dyes to minimize the potential for emission spectrum overlap. The PVL probe was synthesized with the nonfluorescent quencher BHQ3 (TIB Molbiol LLC, Adelphia, N.J.). Reactions were performed with 1× QuantiTect multiplex PCR master mix (QIAGEN, Inc., Mississauga, Ontario, Canada) under the following thermal cycling conditions: 15 min at 95°C, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. Unlabeled primers were synthesized by Sigma Genosys (Oakville, Ontario, Canada). All oligonucleotide sequences and their corresponding reaction concentrations are shown in Table 1. Amplification was confirmed by the generation of a sigmoid amplification plot. The PVL TaqMan PCR assay was validated by comparing results to those obtained by using primers *luk-PV 1* and *luk-PV 2* with a conventional PCR protocol (11).

To validate the PVL TaqMan primer and probe sequence design, comparative results using the conventional PCR and the single-target TaqMan assay were collected for 103 clinical MRSA isolates. Results obtained for these isolates by use of the two methods were in agreement (100% sensitivity, 100% specificity), with 54 isolates yielding positive amplification.

During a 15-month period, 752 clinical MRSA isolates were tested by the single-target PVL assay in conjunction with the duplex assay for *mecA* and *nuc*, of which 321 (43%) were PVL positive. The performance of the triplex TaqMan assay was subsequently compared directly to that of the single-target PVL assay by using the results obtained from 98 different isolates. The results of the two TaqMan assays showed 100% concordance. Reference strains not containing PVL (MRSA [ATCC 43300], *S. aureus* [ATCC 29213], and methicillin-sen-

sitive *S. epidermidis* [ATCC 12228]) did not show amplification with the PVL primers and probe. Reproducibility of the triplex PVL assay was confirmed with the consistent amplification of a clinical isolate of *S. aureus* (BA37594) on 31 separate occasions. Subsequently, 516 consecutive clinical isolates have been assayed with the triplex PCR. PVL was detected in 259 (50%) of these isolates.

CA-MRSA is rapidly becoming a public health concern in many parts of the world, including Canada (7, 13, 21). In Saskatchewan, CA-MRSA, described as similar to USA400 by pulsed-field gel electrophoresis and harboring staphylococcal chromosome cassette *mec* (SCC*mec*) type IV and PVL, has been identified in certain communities (13), and this strain is now widespread in the province (R. R. McDonald, K. Keller, L. Snook, E. Nagle, C. Selin, M. Mulvey, N. Antonishyn, P. N. Levett, and G. Horsman, abstr. 72nd CACMID Conjoint Meet. Infect. Dis., abstr. LB7, 2004). Most infections with PVL-producing CA-MRSA involve skin lesions, but there is also a proportion that present as rapidly progressive necrotizing pneumonia (22, 23). Thus, PVL is thought to be an important virulence factor in these infections as well as a stable marker for CA-MRSA (19). For these reasons, it is important for clinicians to be familiar with the incidence of CA-MRSA in their communities, as prevalence can vary from region to region. Many CA-MRSA soft tissue infections can be treated with incision and drainage, without antibiotic treatment (10). Moreover, since CA-MRSA has a unique antibiotic resistance profile (13, 21), knowledge of prevalence can inform empirical antibiotic therapy in more-severe cases that are handled as outpatients (3). This necessitates surveillance for PVL-containing MRSA by use of the most efficient laboratory methods available.

Real-time PCR allows for rapid detection of target sequences with high throughput and is the best-suited technology for this and other applications. A design of a FAM/VIC/TET triplex PCR on an Applied Biosystems 7700 instrument was contemplated; however, because the potential for cross talk

due to emission spectrum overlap for these three dyes was high, the protocol was abandoned. Indeed, following amplification we observed the detection of fluorescence in the TET (PVL) layer of PVL-negative MRSA isolates. Furthermore, fluorescence was detected in the TET layer of MRSA reactions containing only FAM- and VIC-labeled probes (data not shown). Contrary to another report (14), the reproducibility of these observations did not allow us to pursue a FAM/VIC/TET triplex PCR application. Use of Cy5 as a reporter dye with the Applied Biosystems 7500 instrument was an alternative approach. The change has allowed the assay for *mecA*, *nuc*, and PVL to be less laborious, using only one master mix that also generates savings in costs associated with the assay.

PVL-containing MRSA is an emerging pathogen in the province of Saskatchewan and elsewhere in Canada. The multiplex reaction described in this report should allow rapid detection of CA-MRSA strains by using the presence of the PVL toxin as a marker. In addition, this assay could be used to assess the prevalence of the PVL toxin genes in the general *S. aureus* population.

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